Reference: *Biol. Bull.* **193:** 381–392. (December, 1997)

Symbiosis of the Hydrothermal Vent Gastropod *Ifremeria nautilei* (Provannidae) With Endobacteria—Structural Analyses and Ecological Considerations

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Abstract. The gastropod *Ifremeria nautilei* lives in high abundance around deep-sea hydrothermal vents of the Western Pacific. The filaments of its ctenidium are very long and have a rigid axis with a hemocoelic vessel and a strongly ciliated epithelium. The flattened part of each filament largely consists of bacteriocytes that are distally filled with numerous gram-negative bacteria. The bacteria lie one by one in vacuoles that seem to be part of an interconnected tubular system. Some of the apical vacuoles regularly showed what could be openings to the ambient seawater. This special topological arrangement of the bacteria suggests that in a morphological series mirroring the supposed evolutionary pathway from extra- to intracellular symbioses, *I. nautilei* might correspond to an intermediate stage.

The high sulfur content and the low stable carbon isotope values measured in this study, combined with corresponding data from the literature, indicate that *I. nautilei* is the host partner in a thiotrophic chemoautotrophic bacterial symbiosis. The importance of this symbiosis for the nutrition of the gastropod is underlined by the reduced size of the host's stomach. Unlike specimens of *I. nautilei* from the Manus Basin (Galchenko *et al.*, 1992), the inspected specimens from the North Fiji Basin did not contain any methanotrophic bacteria in addition to the thiotrophic type. From the disparity in results, it may be concluded that this host species can develop different patterns of symbiosis either as an adaptation to local variances of hydrothermal vent fluid chemistry or as a consequence of genetic differentiation in the host.

Received 10 April 1996; accepted 15 August 1997.

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Introduction

Hydrothermal vents, which have been known since the late 1970s, have become the subject of intensive study. Biological investigations have concentrated on vents in the East Pacific and on the Mid Atlantic Ridge, where vestimentiferans, bivalve molluscs, and shrimps dominate the ecosystems (Van Dover, 1990; Tunnicliffe, 1991). It has been shown that symbioses between most of these animals and chemoautotrophic bacteria are the main source of nutrition (Fisher, 1990; Childress and Fisher, 1992). The animals supply the bacteria with reduced compounds from the vents and with oxygen from the ambient seawater. The bacteria gain energy by oxidizing sulfur species—and sometimes methane as well (Childress *et al.*, 1986; Fisher *et al.* 1987, 1993; Cavanaugh *et al.*, 1987; Distel *et al.*, 1995)—for the fixation of organic carbon which, in turn, is utilized by their hosts.

Only in hydrothermal vent ecosystems of the Western Pacific are gastropod molluscs abundant, even attaining a dominant ecological role (Van Dover, 1990; Tunnicliffe, 1991). To date, vent areas have been investigated in the Lau Basin (Desbruyères *et al.*, 1994), the Manus Basin (Both *et al.*, 1986; Tufar, 1990; Galkin, 1992), the Mariana Trough (Hessler *et al.*, 1988; Hessler and Lonsdale, 1991), and the North Fiji Basin (Jollivet *et al.*, 1989;

Desbruyères *et al.*, 1994). In almost all these areas, one or both of the two prosobranch gastropods *Ifremeria nautilei* and *Alviniconcha hessleri* (Family Provannidae) occur in abundance together with several mytilid bivalve species of the genus *Bathymodiolus* (Warén and Bouchet, 1993; Desbruyères *et al.*, 1994; Cosel and Métivier, 1994). The gastropods settle in the vicinity of hydrothermal emissions where they are exposed to temperatures of about 3°–20°C.

Alviniconcha hessleri was the first hydrothermal vent gastropod reported to contain endosymbiotic bacteria within its specialized gills (Ohta *et al.*, 1988, Stein *et al.*, 1988; Endow and Ohta, 1989a, b). The high content of elemental sulfur, the autotrophic fixation of CO_2, and the high activity of enzymes that catalize sulfide oxidation indicated that the bacteria were chemoautotrophic sulfur-oxidizing endosymbionts (Stein *et al.*, 1988). But Ohta *et al.* (1988) and Endow and Ohta (1989a, b) found in one of the three specimens of *Alviniconcha hessleri* inspected a second morphological type of bacterial symbionts closely resembling methanotrophic bacteria.

In micrographs of *A. hessleri* (Stein *et al.*, 1988), bacteria appear to be intracellular and enclosed singly in host vacuoles, although some vacuoles with several bacteria can be seen. But Endow and Ohta (1989a, b) noted that the apical vacuoles of several bacteriocytes contained narrow ducts that seemed to directly connect the bacteria to the exterior. On the other hand, they demonstrated by infiltration of ruthenium red that in the basal parts of the bacteriocytes the symbionts were truly enclosed in their vacuoles (Endow and Ohta, 1989b).

Ifremeria nautilei, the other hydrothermal gastropod species from the Lau and North Fiji Basins (Bouchet and Warén, 1991; Beck, 1991; Warén and Bouchet, 1993), deviates in several anatomical details from the related provannid *A. hessleri*. The hypertrophic gills, the well-developed circulatory system, and the reduced stomach lead to the assumption that symbiotic bacteria might play a more significant role in these animals. Galchenko *et al.* (1992), examining specimens from the Manus Basin, provided the first physiological evidence for a bacterial symbiosis. CO_2-fixation was stimulated by reduced sulfur compounds, and radiolabeled CH_4 was metabolized and detected in the tissues. These results suggested the presence of thioautotrophic and methanotrophic bacteria in the gills, providing the gastropod with reduced carbon. The ultrastructural basis given by the authors for these results was very preliminary, and the number of specimens or ctenidial filaments inspected was not indicated. The micrographs supported the physiological data, reinforcing the conclusion that two different physiological pathways were being used by two morphologically distinct types of bacteria. Specimens of *I. nautilei* from the Lau and North Fiji Basin have only been studied physiologically: Desbruyères *et al.* (1994) confirmed the presence of thiotrophic symbionts by the detection of ribulose-1, 5-bisphosphate carboxylase activity.

The present study on *Ifremeria nautilei* from the North Fiji Basin concentrates on an (ultra)structural survey of the bacterial symbiosis to scrutinize important details previously not sufficiently documented (Galchenko *et al.*, 1992). These structural results are supplemented by measurements of elemental sulfur, total sulfur, and stable carbon isotope composition. They give further insight in the trophic interactions between the endosymbiotic prokaryotes and their gastropod host.

Materials and Methods

During cruise 99 of the RV *Sonne* in January 1995 (Auzende *et al.*, 1995; Halbach *et al.*, 1995), many specimens of *Ifremeria nautilei* (Bouchet and Warén, 1991) were collected in the North Fiji Back Arc Basin at the LHOS site (Ishibashi *et al.*, 1994) at 16°59.65 S–173°54.73 E. The sample was retrieved from a depth of 2000 m (station GTV 115) by a TV-controlled grab. The grab also contained the symbiont-harboring bivalve *Bathymodiolus brevior* and other hydrothermal animals. The specimens of *I. nautilei* examined had a shell height of about 5 cm. Five gill filaments from each of two specimens were dissected and fixed in a mixture of 4% paraformaldehyde, 3% glutaraldehyde, and 15% sucrose in 0.1 *M* cacodylate buffer (pH 7.4). After being rinsed in the same buffer, the tissue was postfixed in OsO_4 (1%). Samples were dehydrated in acetone, embedded in Spurr's resin, and sectioned for light and electron microscopy. For light microscopy, semithin sections were stained with toluidine blue; for electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate. Sections of filament from the two specimens were examined using a Zeiss 902A electron microscope. One filament was sequentially cut and, in intervals of 100 μm, ultrathin sections were examined. For scanning electron microscopy, several gill filaments of two formaldehyde-fixed (4%) specimens were dehydrated in acetone, critical-point-dried, and gold-sputtered. A Cambridge Camscan DV 4 scanning electron microscope was used for the observations. For paraffin sections, a complete specimen was fixed in 4% formaldehyde. After dissection and standard embedding, sections were cut and stained with Azan (Romeis, 1989).

Measurements of total sulfur (S^{total}) concentration were based on samples from three individuals (10 to 40 mg dry weight). The lyophilized gill and muscle tissues of the foot (excluding the warts, Warén and Bouchet, 1993) were digested separately for 3 h under pressure at 130°C

in 4 ml HNO_3 (65%, suprapur, Würfels and Jackerth, 1985). The resulting solution was concentrated by evaporation to 0.5 ml, then brought to 14 ml with distilled water. In this solution the sulfur concentration was measured with an inductivity-coupled-plasma atomic emission spectrometer (ICP-AES, *Perkin Elmer Plasma II*). Oyster tissue SRM 1566, commonly used in mollusc studies, and a diluted standard solution were used for calibration.

For measurements of elemental sulfur (S^0), frozen gill and foot muscle tissues of three individuals were lyophilized and analyzed by HPLC (pump: Jasco 880 PU, manual injector: Rheodyne 7105, fitted with a 20 μl loop; UV/VIS detector: Jasco 975-UV, set at 254 nm; column: Hamilton PRP-1 reversed phase (15 cm × 4.1 mm I.D.). The absorbance was continuously collected by a computer using the Labtech notebook software. Chromatograms were analyzed using the Labtech chrom software. Treatment of the column prior to analysis, extraction of samples in chloroform, and HPLC-protocol were according to Lauren and Watkinson (1985).

The $\delta^{13}C$ was measured with a Finnigan Delta E mass spectrometer from gill tissue of three specimens. After decalcification with 0.1 N HCl and drying in an oven at 60°C, samples were combusted, at 1050°C in a stream of oxygen, to CO_2 by using a commercial Heraeus CHN analyzer attached to the mass spectrometer. Stable isotope determinations were expressed as the permille difference from the international PeeDee Belemnite standard, where $\delta^{13}C = [Ratio_{sample} \times Ratio_{standard}]^{-1} \times 10^3$ (‰).

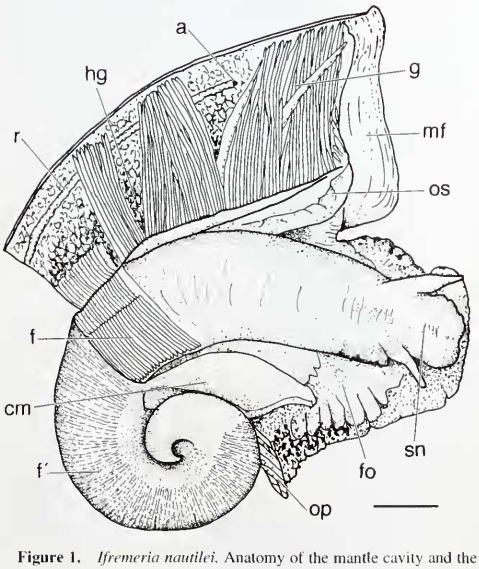

Figure 1. *Ifremeria nautilei*. Anatomy of the mantle cavity and the ctenidium to show the dimension and location of the ctenidium. The shell has been removed, the mantle cavity partly opened, and in "f" the basal attached part of the filaments dissected from mantle. a, anus; cm, columellar muscle; f, gill filaments; f', gill filaments inside the mantle cavity; fo, foot; g, gill; hg, hypobranchiat gland; mf, mantle fringe; op, operculum; os, osphradium; r, rectum; sn, snout. Scale 10 mm (after Beck, 1991, modified).

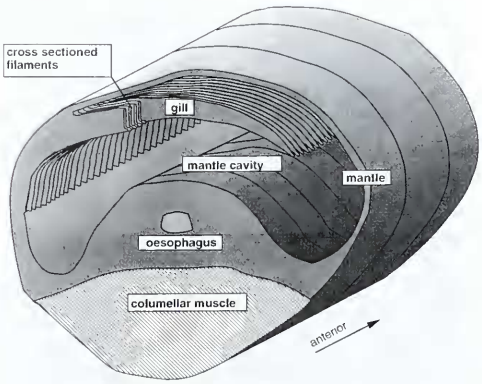

Figure 2. Schematic drawing of interior anatomy showing position and arrangement of the ctenidium in the mantle cavity.

Results

Anatomy of the gill

Ifremeria nautilei has a single ctenidium at the left side of its body, where it occupies about one whorl of the snail's shell (Fig. 1). The ctenidium consists of 1000 to 2000 serially arranged slender filaments with a length of 9 to 17 mm and a maximal width of about 0.1 to 0.2 mm. Towards their proximal and distal ends, these filaments decrease in length and width. The basal third of each filament is attached to the roof of the mantle cavity and the remaining two thirds extend free into the mantle cavity (Fig. 2). The leaflike main part of the filament is thin, its free lower edge, orientated towards the mantle cavity, is reinforced by a rigid axis (Fig. 3A). In some preparations the axis was found bent towards the anterior end of the animal, resulting in an L-shaped filament (Fig. 4); this curvature may be dependent on muscle contraction or fixation. The following description is based on cross sections through the nonattached part of a ctenidial filament.

Each filament consists of two epithelial cell layers separated by a bilayered middle lamella of noncellular material (Figs. 3, 4). The internal space between these lamellar

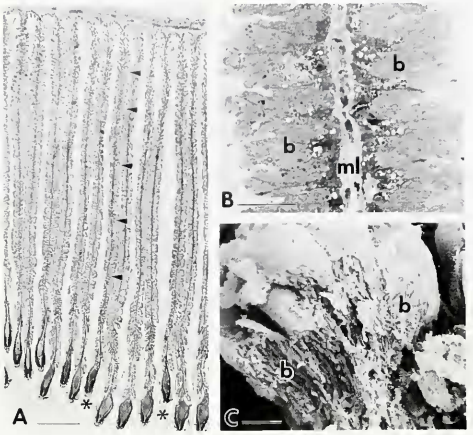

Figure 3. (A) Cross section through several attached ctenidial filaments. Nearly the entire filament consists of bacteriocytes (arrowheads). The ventral edge (*) contains a wide hemocoelic vessel; the dorsal edge is attached to the mantle (azan-stained paraffin section, light micrograph). Scale 1 mm. (B) Details of filament, median cross section, showing the two layers of bacteriocytes (b) separated by the middle lamella (ml). Scale 100 μm. (C) Scanning electron micrograph of bacteriocytes with cell wall peeled open showing the dense packing of bacteria (b). Scale 5 μm.

layers (width varying between 2 and 15 μm) harbors muscles, nerves, connective tissue, hemocoel with hemocytes, and additional extracellular material. At the distal, ventral edge of the filament the two layers of extracellular material become very thick (50 μm) and join, enclosing a wide hemocoelic vessel that extends all along the lower edge of the ctenidial length. In fixed material the hemocoelic space appears more or less empty, containing only some granular material, possibly remnants of the blood fluid (Fig. 5A). At the boundary walls, very flat cells are abundant (Fig. 5C).

Each cross section through a filament (dorsal to ventral, see Fig. 2) can be subdivided into six zones, five of which represent the symbiont-free parts, the remaining large one the symbiont-containing median part of the ctenidial filament (for numbering see Fig. 4). Zones 1–4 form the lower (ventral) axis of the filament, zone 6 the upper (dorsal) edge.

Symbiont-free parts. The five zones that lack symbionts are described below.

Zone 1—the ventral edge of the filament (Fig. 5A). It consists of a ciliated epithelium with cells having a height of 10 to 20 μm and containing large nuclei (5 μm long). Externally, they are covered by 1.25 μm long microvilli and by several 5 μm long cilia. Glandular cells are abundant and contain electron-lucent vacuoles that harbor several homogeneous granules. Underneath the epithelium several small nerves traverse the filament (arrows in Fig. 5A).

Zone 2—the zone enclosing the vessel (Fig. 5B). Here, flat (about 4 μm high) nonciliated epithelial cells form a very thin cellular cover on the extracellular material. The cells contain only little electron-dark cytoplasm; at their external side they are studded with microvilli 0.5 to 0.7 μm in length. The mitochondria are electron dense, their cristae rarely visible.

Zone 3—the zone of strong ciliation (Fig. 5C). It consists of two layers of large, strongly ciliated cells (15 μm high), based on thin (1 μm) noncellular lamellae that en-

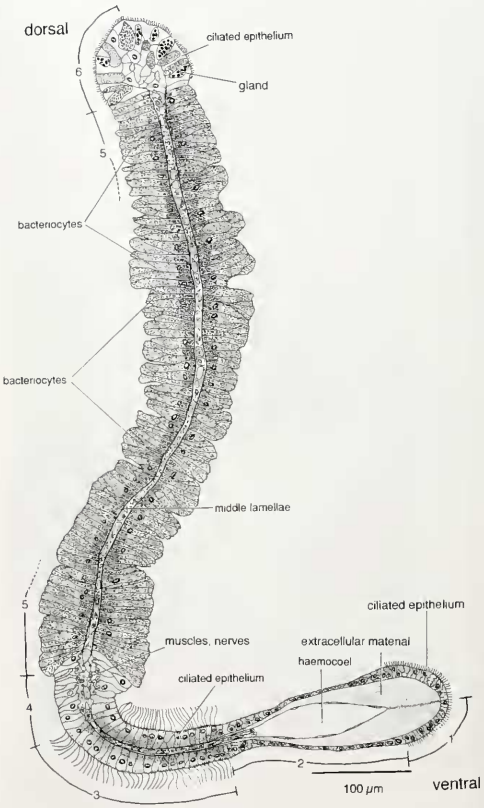

Figure 4. Median cross section of a ctenidial filament. Drawing compiled from light and electron microscopic observations. Bacteriocytes are the dominant cell type. Numbers indicate "zones" of different epithelia as described in the text.

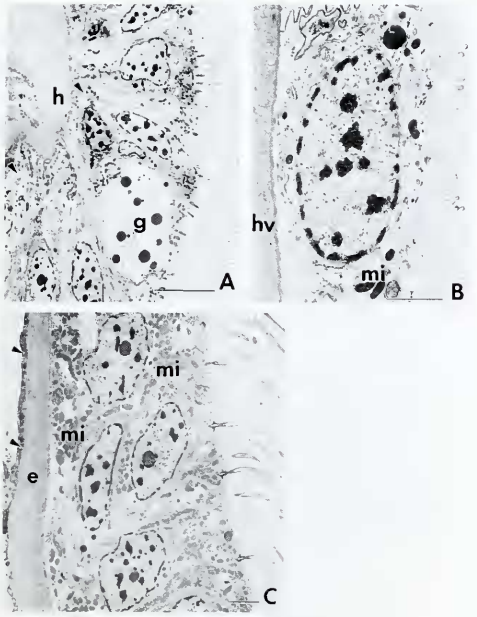

Figure 5. Cross section through ctenidial filament with various types of epithelia; see Figure 4 for zone designation. (A) Zone 1. Ciliated epithelium from the ventral edge of a filament with a gland cell (g). h = hemocoel, arrowheads = nerves. Scale 5 μm. (B) Zone 2. Flat, nonciliated epithelium covers the hemocoelic vessel (hv). Mitochondria (mi) have, as in all other cells of the gill, a very electron-dark matrix. Scale 2 μm. (C) Zone 3. Strongly ciliated epithelium with many mitochondria (mi) and an underlying thick layer of extracellular material (e), arrowheads = thin layer of flat cells. Scale 1 μm.

close a separating space about 2 μm in width. This lacunar space is nearly completely filled with cells that probably represent hemocytes (not shown in micrograph). In each ciliated cell three areas can be recognized: The apical area, 3.5 μm high, is characterized by abundant cilia up to 20 μm long. Here, in addition to mitochondria, the cytoplasm contains rough endoplasmatic reticulum and spherical, electron-dense vacuoles 0.5 μm in width. The middle area of the cells is dominated by globular, large nuclei of about 3.5 μm diameter. The basal cell area is completely filled with tightly packed oval mitochondria. These are about 0.5 μm in length and contain many parallel, well-developed cristae.

Zone 4—the hinge zone (Fig. 6A). Located between the ciliated cells (zone 3) and the bacteriocytes (zone 5), this region is characterized by large (15 μm high), nonciliated cells. In comparison to the surrounding tissue, here both the cell membranes and the thin (0.5 μm) extracellular lamellae are strongly folded. This might indicate the function of this zone as a hinge between the axis and the rest of the filament. The two central lamellae are separated by an interlamellar space that is about 15 to 20 μm in width and traversed by two groups of muscles, two large nerves, and a hemocoelic lacuna. Minor nerves are located at the base of the epithelial cells.

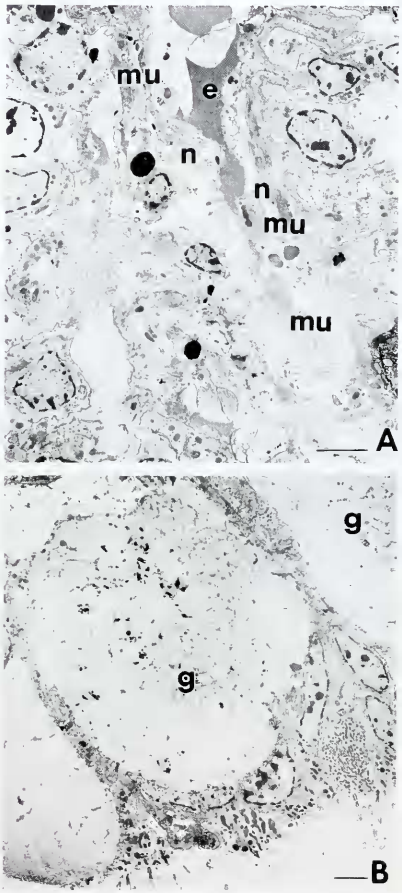

Figure 6. Cross section through ctenidial filament with various types of epithelia, continued; see Figure 4 for zone designation. (A) Zone 4. A "hinge" epithelium with a well-developed middle lamella in the center where nerves (n), muscles (mu), and extracellular material (e) are abundant. Scale 2 μm. (B) Zone 6. Ciliated epithelium with prominent gland cells (g). Scale 2 μm.

Zone 6—the upper filament edge (Fig. 6B). In the nonattached part of the ctenidial filament this zone forms the dorsal edge, characterized by an epithelium consisting of glandular and ciliated cells. Three morphotypes of glandular cells with apparently different secretions can be distinguished. Interspersed between these glandular cells are intercalary, multiciliated cells with cilia 5 μm long.

Bacteriocytes and bacterial symbionts in ctenidial filament. The symbiont-containing median part of the ctenidial filament is Zone 5.

Zone 5—the symbiont zone (Figs. 3, 4, 7A, 9). Representing nearly the entire flattened part of the filament, this dominant region is characterized by numerous symbiont-containing epithelium cells (bacteriocytes). About 250 of these cylindrical to pear-shaped bacteriocytes (about 30 to 50 μm long, maximum diameter about 8 μm) are visible on each cross section through the filament.

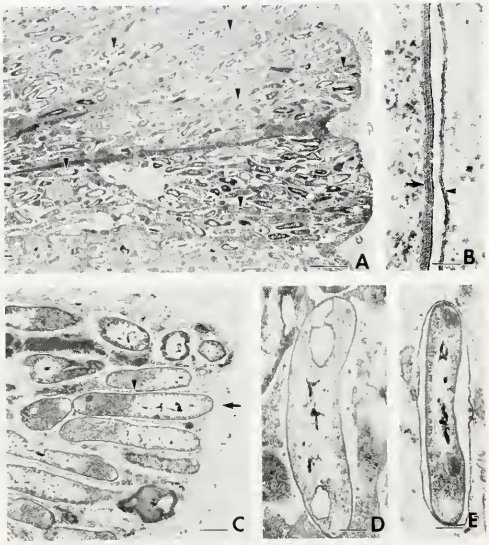

Figure 7. (A) Longitudinal section of the distal part of a bacteriocyte demonstrating the dense packing of bacteria (arrowheads). Only one morphological type of bacterium is observed. Scale 2 μm. (B) Cell envelope of the bacterium with a typical gram-negative pattern (arrow) and the adjacent unit membrane (arrowhead) of the host cell. Scale 50 nm. (C) Typical parallel arrangement of bacteria in the apical region of a bacteriocyte. Note anastomoses in vacuoles containing bacteria (arrowhead) and the opening of one vacuole to the outside (arrow). Scale 0.5 μm. (D) Bacterium containing membrane-bound electron-lucent vacuoles. Scale 0.25 μm. (E) Rod-shaped bacterium with peripherally arranged granular plasma and centrally condensed DNA, but without vacuoles. Scale 0.25 μm. Micrograph D is from a specimen with high S^0 content in the gill; E from a specimen with low S^0 in its gill.

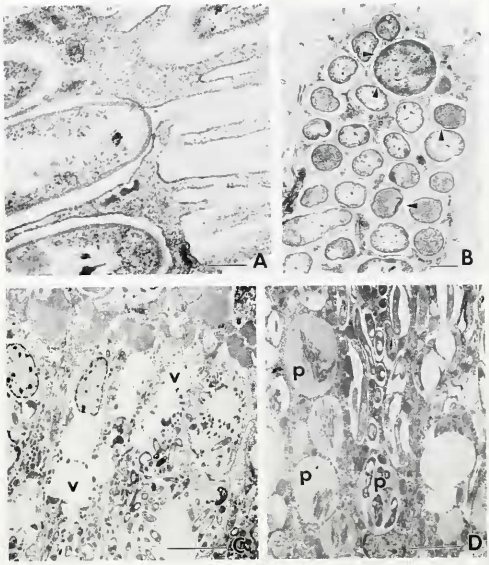

Figure 8. (A) Opening of a bacterium-containing vacuole to the outside of the cell. Scale 0.2 μm. (B) Apical tip of bacteriocytes in cross section showing anastomoses (arrowheads) between the bacterium-containing vacuoles. Scale 0.5 μm. (C) Basal part of bacteriocytes. Abundant non-membrane-bound electron-lucent vacuoles (v) whose content might have been partly extracted during preparation. Scale 5 μm. (D) Basal part of bacteriocytes. Structures resembling phagolysosomes (p) in different stages. Scale 2 μm.

The cytological structure of the bacteriocytes is fairly uniform. Their surface is studded with 0.7 μm-long microvilli. The outer parts of these cells are densely filled with inclusions that resemble bacteria (Figs. 3C, 7, 8). Most are about 2.5 μm (length) by 0.5 μm (width), but some may reach a size of 4 μm by 1 μm. Their cell envelope is of the gram-negative type with clearly visible inner and outer membranes (Fig. 7B). A peripherally located dense cytoplasm and a central network of condensed DNA betray the prokaryotic nature of these cells (Figs. 7C, D, E). In one of the two specimens inspected the bacteria contained membrane-bound electron-lucent vesicles (Fig. 7D). Typical bacterial division stages were not found. Since transitional stages between all bacterial sizes were found, there are no reasons to assume the existence of different morphological types of bacteria in the examined sections. Membrane stacks, characteristic for the methanotrophic bacteria, could never be discerned.

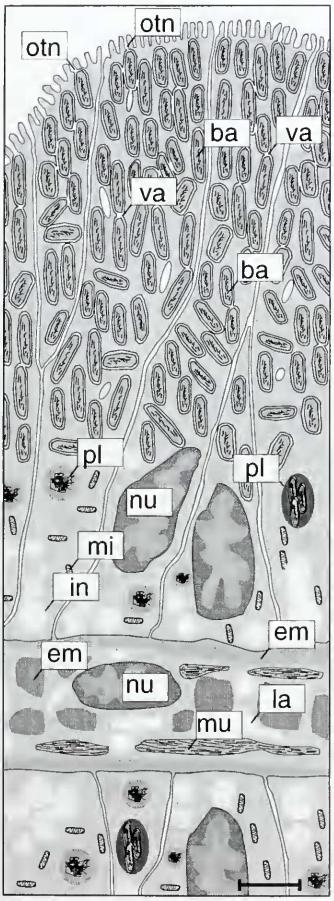

Figure 9. Schematic representation of bacteriocytes and underlying middle lamella. ba, bacteria; em, extracellular material; in, electron-lucent inclusion; la, hemocoelic lacuna; mi, mitochondrium; mu, muscle; nu, nucleus; otn, opening of apical bacterial vacuoles; pl, phagolysosome-like structure; va, bacteria-containing vacuoles. Scale 5 μm.

Each bacterium was tightly embedded in a vacuole, but the vacuoles often appeared interconnected. Therefore there seems to exist a complex "network" of anastomosing vacuoles harboring the bacteria. (Figs. 7C, 8B). Moreover, in all the examined sections, some peripheral vacuoles were observed to have an opening towards the outside environment (Figs. 7C, 8A).

Whereas the distal part of the bacteriocytes contained only bacteria and a few mitochondria, the basal part was dominated by the nucleus and several large, non-membrane-bound droplets of a homogeneous, electron-lucent material (Fig. 8C). This internal cell area also harbored many 1 μm-long electron-dark mitochondria that were rich in cristae. Inclusions resembling phagolysosomes (Fiala-Médioni *et al.,* 1994), which would represent stages of bacteria in digestion, could often be observed in the basal part of the bacteriocytes (Fig. 8D).

Interspersed between the bacteriocytes, but in much lower abundance, were secretory cells and a few intercalary cells. The two lamellae between the epithelial layers of bacteriocytes enclosed a space that was filled by hemocoel, putative hemocytic cells, thick cords of collagen, and connective tissue (Figs. 3B, 6A).

Sulfur and ^{13}C measurements

In the gill and foot tissue of *Ifremeria nautilei,* sulfur was detected (Table I) in concentrations comparable to those in other symbiont-containing molluscs, but somewhat lower than those reported for Vestimentifera (see compilation in Stein *et al.,* 1988). Concentration of elemental sulfur (S^o) differed by a factor of 8 among the individuals examined. The average concentrations of S^o and S^{total} were significantly higher in the gill than in the foot (Table I).

The stable carbon isotopic values measured from three specimens of *I. nautilei* equaled −31.4‰, −31.4‰, and −33.7‰. Thus, they were much more depleted than those in marine animals that are linked to a photosynthetically based food chain (Fry and Sherr, 1984).

Table I

Percentages (±SD) of elemental sulfur (S^o) and total sulfur (S^{total}) (based on mg dry weight) in gill and foot of three individuals (115-1, 115-2, 115-4) of Ifremeria nautilei

Sample	Organ	S^o	S^{total}
115-1	gill	0.54 ± 0.02	2.23
	foot	0.25 ± 0.21	1.43
115-2	gill	3.98 ± 0.93	2.49
	foot	0.13 ± 0.13	1.14
115-4	gill	1.88 ± 0.14	2.84
	foot	0.03 ± 0.01	1.05

Values for S^o and S^{total} are significantly higher in the gill than in the foot (Mann-Whitney *U*-test). In gill sample 115-2, the higher value of S^o than S^{total} may account for the patchy localization of elemental sulfur in the tissue. Additionally, the resulting variance of data may be altered by the different sample sizes required for the two methods of sulfur identification.

Discussion

Comparison with other provannid gastropods

The first hydrothermal vent gastropod shown to contain thiotrophic bacteria in its gills was *Alvinoconcha hessleri* (Stein *et al.*, 1988). This provannid species is often found around the same hydrothermal outlets as *Ifremeria nautilei*, to which it is closely related (Both *et al.*, 1986; Jollivet *et al.*, 1989; Tufar, 1990; Galchenko *et al.*, 1992; Desbruyères *et al.*, 1994). Despite some differences in gross morphology—*e.g.*, in shell structure, nervous system, and pallial margin, as well as in the presence of oesophageal pouches and wartlike tubercles on the foot of *L. nautilei* (Warén and Bouchet, 1993)—the two species apparently have a very similar type of bacterial symbiosis. This conclusion is based on ultrastructural data from the preliminary work by Galchenko *et al.* (1992) and from the present study.

An array of structural features of *I. nautilei* indicates that the bacterial symbiosis is of high functional importance for the host.

- The most obvious trait is the extremely high number and unusual length of ctenidial filaments. *I. nautilei* has 15 to 20 times more ctenidial filaments than the 30 to 89 found in its aposymbiotic relative *Provanna* spp. (Warén and Ponder, 1991).
- The ctenidial filaments in monopectinate prosobranch gastropods are usually triangular, with their base fully attached to the mantle roof (Demian, 1965). A ciliary ridge extending from the efferent edge of the filaments creates water currents for irrigation (Lutfy and Demian, 1965). In *I. nautilei* this efferent edge is highly elongated and a rigid axis has developed that bears the extended bacteriocytic epithelia and encloses a wide hemocoelic vessel. This axis may enhance the stability of the flimsy leaflike filaments. It also may act as a flexible hinge that, in cooperation with longitudinal and transverse muscle fibers, would allow movement of the whole filament. The "blood" vessel is presumably responsible for nutrition and gas exchange.
- The bacteria occur in extremely high numbers in the bacteriocytes and occupy a large area of the filaments. The stomach of the host is reduced to only $^1/_{10}$ the size of the stomachs of related nonsymbiotic provannid gastropods (Bouchet and Warén, 1991). This points to the relevance of the symbiosis for the animals' nutrition. Furthermore, even though bacteriocytes represent the dominant structures in the gill tissues of other molluscs with a bacterial symbiosis, the bacterial zone in *I. nautilei* is nearly twice as voluminous as in these species (Le Pennec and Hily, 1984; Southward, 1986; Fiala-Médioni *et al.*, 1986; Fiala-Médioni and Métivier, 1986; Fiala-Médioni, 1988; Fiala-Médioni and Felbeck, 1990). Whether this difference mirrors a larger dependence on chemoautotrophic nutrition is an open question.

The black-tipped warts on the foot of *I. nautilei* have been suspected, without closer analysis, to be "involved" in the symbiosis (Warén and Bouchet, 1993). Our analyses of ultrathin sections through the warts (data not shown) did not demonstrate the presence of any bacteria, thus refuting such an assumption.

Position of the bacteria in a vacuolar network

In most molluscs with chemoautotrophic bacteria, the prokaryotes are intracellular although their detailed arrangement may vary. In *I. nautilei* the position of the symbiotic bacteria in vacuoles of ctenidial cells is essentially the same as that described by Endow and Ohta (1989a, b) for the related gastropod *A. hessleri*. These studies indicate that peripheral vacuoles have some contact to the ambient seawater via small ducts. This topological arrangement seems to be specific to these two related gastropod species and might indicate a common origin of the symbiosis. In *A. hessleri* it could be demonstrated by staining with ruthenium red that this connection via "open" vacuoles was restricted to the externally located bacteria (Endow and Ohta, 1989b). Regrettably, the fresh material needed for this experiment was not retained in *I. nautilei*.

The chemoautotrophic nature of the bacteria

In specimens of *I. nautilei* from the Lau and Fiji Basins (Desbruyères *et al.*, 1994) and from the Manus Basin (Galchenko *et al.*, 1992), ribulose-1, 5-bisphosphate carboxylase/oxygenase activity provided evidence for the chemoautotrophy of the symbiotic bacteria. The stable carbon isotope values measured for *I. nautilei* in this study are higher than those reported for specimens from the Manus Basin (Galchenko *et al.*, 1992). They stay in the range of values found in symbiont-containing bivalves known to depend fully on their symbionts for nutrition (Childress and Fisher, 1992). Supporting evidence for the thiotrophic nature of the symbionts is provided by the high concentration of sulfur in the gills; this is regarded as a reliable criterion for the presence of sulfur-oxidizing bacteria (Childress and Fisher, 1992). It is, of course, still possible that this sulfur is a product of purely chemical oxidation of sulfide. The concentration of elemental sulfur in *I. nautilei* (0.5%–3.9% dry weight) is comparable to that in the gastropod *A. hessleri* (Stein *et al.*, 1988) or in the bivalves *Lucinoma annulata* (0.2%–2.1% dw; Vetter, 1985) and *Calyptogena magnifica* (4.4% dw, Stein *et al.*, 1988). Similarly, the concentration of total sulfur (2.2%–2.8% of dry weight) in the gills of *I. nautilei* is in the same range as in the gills of *L. annulata* (2.5%–5.6%

dw; Vetter, 1985) and *C. magnifica* (3.8% dw; Roesijadi and Crecelius, 1984) which have been proven to harbor thiotrophic bacteria. The foot, which has never been found to harbor bacteria in any mollusc with bacterial symbionts, contained about 1% dry weight total sulfur in *I. nautilei* (see Table 1).

The regular occurrence of degradation stages of symbiotic bacteria could indicate a digestive utilization of the prokaryotes by their host. This "harvesting" via phagocytotic degradation has also been found in *Riftia pachyptila* (Bosch and Grassé, 1984), in symbiont-harboring oligochaeta (Giere and Langheld, 1987), and in bivalves (Fiala-Médioni and Felbeck, 1990).

Thiotrophic vs. *methanotrophic bacteria*

Methanotrophic bacteria have been reported in *I. nautilei* from the Manus Basin (Galchenko *et al.*, 1992). The identification was based on the activity of the methanol-oxidizing enzyme methanol dehydrogenase, the incorporation of labeled carbon from methane, the very low $\delta^{13}C$ value of the gills (−38.3‰), and the presence of intracytoplasmic membranes in some of the bacteria. This is in contrast to the present study on specimens of the same species retrieved in the Fiji Basin, some 3000 km away. We could not discover any bacteria with the internal membranes typical of methanotropic bacteria (Childress *et al.*, 1986; Fisher *et al.*, 1987; Cavanaugh *et al.*, 1987; Cavanaugh, 1992; Fisher *et al.*, 1993; Distel *et al.*, 1995). Although, at least in free-living methanotrophs, the occurrence of membranes is variable depending on culture conditions (Prior and Dalton, 1985; Collins *et al.*, 1991), the higher $\delta^{13}C$ values found in our specimens might also indicate an absence of methanotrophs. For a more conclusive interpretation, the $\delta^{13}C$ values of the methane sampled at the North Fiji hydrothermal vents are required.

Our samples, taken from only a few specimens and ctenidial filaments, might have missed material containing methanotrophic bacteria due to an inhomogeneous distribution in the large gills. Alternatively, the small-scale spatial and temporal variability in the physical-chemical composition of the environment (Chevaldonné *et al.*, 1991) might have caused the gastropod population to show local variations in bacterial symbiont stock. Both options have also been considered by Ohta *et al.* (1988), who postulated that methanotrophic symbionts were present only in low abundance (up to 10% of the symbionts). They also discussed the possibility that the methanotrophic bacteria might not be present in all members of the gastropod population, depending on the ambient conditions. Endow and Ohta (1989b) suggested that the putative methanotrophic symbionts of *A. hessleri*, as well as the prevailing thiotrophic type, might have been acquired "horizontally" from the outside. This would relate the symbiosis to environmental conditions, *i.e.*, the presence of the symbionts in the environment. From an adaptive perspective, it would lend the host a favorable flexibility to cope with the high spatial and temporal variability of the vent ecosystem and might explain why *A. hessleri* and *I. nautilei* are mixotrophs that have retained a functional, though reduced, digestive tract (Bouchet and Warén, 1991; Warén and Bouchet, 1993; Desbruyères *et al.*, 1994). It could then be assumed that the composition of thiotrophic and methanotrophic bacteria reflects the composition of the reduced compounds released from the vents (Distel *et al.*, 1995). This variability would also explain the inconsistency in our measurements of S^0 in *I. nautilei*, which varied by a factor of 8. These considerations imply that assessment of one or two types of symbionts might depend on fortuitous individual temporal or local conditions. Physiological assessment through diagnostic enzymes might be problematical because low concentrations can escape detection (Endow and Ohta, 1989b). Hence, one probably has to apply molecular techniques to reliably demonstrate the presence or absence of different symbiont types. The occurrence of more than one type of symbiotic bacteria in one host has been shown to exist in several populations of the mytilid bivalves (Childress *et al.*, 1986; Fisher *et al.*, 1987; Cavanaugh *et al.*, 1987; Fisher *et al.*, 1993; Distel *et al.*, 1995) and in gutless oligochaetes (ultrastructural evidence: Giere *et al.*, 1995; molecular evidence: Dubilier *et al.*, unpubl. data).

Genetic differences between specimens from the Manus and the North Fiji Basin might also develop in response to the spatial and temporal instability of hydrothermal vents. Genetic separation in populations of neighboring vent fields has been shown to occur in *Alviniconcha hessleri* (Denis *et al.*, 1993) and in the bivalves *Calyptogena* spp. (Vrijenhoek *et al.*, 1994) and *Bathymodiolus* spp. (Craddock *et al.*, 1995).

Ecological considerations and evolutionary aspects

It is commonly argued that there is an evolutionary trend from extra- to intracellular symbioses (Smith, 1979; Southward, 1986; Craddock *et al.*, 1995; Giere, 1996; Ott, 1996). In gastropods, the first step might be represented by *Lepetodrilus fucensis* from the northeastern Pacific. In all sections of this vent limpet, bacteria originating from the dense colonies of bacteria on the epithelial surface of the gill have been observed to become endocytosed and digested in lysosome-like organelles (De Burgh and Singla, 1984). This has been interpreted as a routine process that occurs in addition to the normal grazing on the ambient bacterial population. In *Alviniconcha hessleri* and *Ifremeria nautilei* the symbiotic bacteria are enclosed

in vacuoles, but still appear to have some contact with the outside. This would represent an intermediate step in a morphological series. The most advanced step, complete incorporation of symbionts into bacteriocytes, is common in many bivalve molluscs, but has not yet been described from symbiont-harboring gastropods.

The bacterial symbioses in *A. hessleri* and *I. nautilei* are structurally very similar, and the two hosts often settle close together in hydrothermal fields. Taxonomically, however, the snails have been assigned to different gastropod genera. This taxonomic difference might reflect a slight ecological or physiological niche diversification. Usually, *A. hessleri* has been found a little closer to the hydrothermal outlets than *I. nautilei* (Bouchet and Warén, 1991; Galchenko *et al.*, 1992; Warén and Bouchet, 1993; Desbruyères *et al.*, 1994). It seems possible that this pattern is temperature dominated and mirrors specific differences in temperature preference. In the North Fiji Basin, Chevaldonné *et al.* (1991) obtained data on temperature gradients and the distribution of the gastropod colonies. But the complex temperature pattern seems to change locally very much and, for the area investigated here, it is not possible to relate the distribution pattern of the two gastropods to the temperature gradients around the hydrothermal outlets.

In molluscs, symbiosis obviously developed several times independently (Distel *et al.*, 1994). The gills of bivalves and gastropods harbor different types of bacteria, sometimes even in an individual host. The present study indicates that separate populations of the same species may differ in their bacterial partners. An intraspecific symbiotic variability between bacterial groups (methanotrophs and thioautotrophs) may have contributed to the success of these gastropods in the unstable and ecologically diverse ecosystems of Pacific hydrothermal vents (Van Dover, 1990). The reasons for their distributional restriction to the Southwest Pacific remain unclear, but a more detailed picture of the symbiotic variability and ecological versatility may help to clarify it.

Acknowledgments

We would like to thank the crew of the RV *Sonne* 99 cruise for their good cooperation. We are indebted to Prof. Dr. W. Dannecker and Dr. F. Meyberg from the University of Hamburg for the ICP-AES analysis, Dr. B. Vismann from the Marine Biological Laboratory in Helsingor for HPLC analysis of S^0, and Dr. M. Segl from the University of Bremen for ^{13}C measurements. We thank G. Wegener for her valuable assistance in the microscopical preparations. Our special thanks go to two anonymous colleagues for their efforts in reviewing the manuscript. Their work has much improved this paper. This study was financially supported by the Ministry of Education, Science, Research and Technology (BMBF) of Germany, grant no. 03G0099A.

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